THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 4, Issue of January 26, pp. 2943–2952, 2001

Printed in U.S.A.

Two Classes of Androgen Receptor Elements Mediate Cooperativity through Allosteric Interactions*

Received for publication, October 6, 2000
Published, JBC Papers in Press, October 30, 2000, DOI 10.1074/jbc.M009170200

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Genes uniquely regulated by the androgen receptor (AR) typically contain multiple androgen response elements (AREs) that in isolation are of low DNA binding affinity and transcriptional activity. However, specific combinations of AREs in their native promoter context result in highly cooperative DNA binding by AR and high levels of transcriptional activation. We demonstrate that the natural androgen-regulated promoters of prostate specific antigen and probasin contain two classes of AREs dictated by their primary nucleotide sequence that function to mediate cooperativity. Class I AR-binding sites display conventional guanine contacts. Class II AR-binding sites have distinctive atypical sequence features and, upon binding to AR, the DNA structure is dramatically altered through allosteric interactions with the receptor. Class II sites stabilize AR binding to adjacent class I sites and result in synergistic transcriptional activity and increased hormone sensitivity. We have determined that the specific nucleotide variation within the AR binding sites dictate differential functions to the receptor. We have identified the role of individual nucleotides within class II sites and predicted consensus sequences for class I and II sites. Our data suggest that this may be a universal mechanism by which AR achieved unique regulation of target genes through complex allosteric interactions dictated by primary binding sequences.

Despite intensive efforts investigating how the androgen receptor (AR)[1] uniquely regulates androgen-responsive promoters, the fundamental mechanisms governing this specificity are still not completely understood. The specificity of steroid receptor response can arise through at least five stratified regulatory mechanisms, including: availability of constituents in the tissue such as hormones, corresponding receptors, and transcriptional coregulator proteins; influence of proximal transcription factors bound to the promoter; cooperative binding to multiple hormone response elements; and sequence-specific DNA target recognition of individual binding sites (reviewed in Ref. 1). For the majority of nuclear receptors, specificity of response is acquired largely by sequence specificity of the primary DNA-binding site. This level of specificity is commonplace with nuclear receptors within the thyroid, retinoid, estrogen receptor (ER) subfamily that bind as homodimers and heterodimers as well as orphan receptors that bind as monomers to distinctive extended DNA sequences (2–7). Recently, evidence has emerged that steroid receptors can also use sequence specificity to a degree to help discriminate their binding targets (1, 8, 9). However, steroid receptors appear to often have overlapping sequence specificity within the primary binding site and must employ additional mechanisms to achieve exclusive regulation of their cognate regulated promoters (10–12). In the case of AR, a number of studies have shown that AR binds cooperatively to multiple androgen responsive elements (AREs) within native promoters, and this is likely a fundamental mechanism for AR-specific transcriptional activation (13–18).

By definition, cooperative DNA binding is the binding of a protein at one DNA site that facilitates the binding of other protein molecules at additional distant sites. This can occur by stabilizing the binding of the resulting complex or by making the additional binding sites more accessible (19–21). Stabilization of a complex typically involves protein-protein interactions and results in distant DNA sites brought together, looping out the intervening DNA and resulting in an interactive cluster of transcription factors. If cooperative binding involves creating access to DNA sites, then it is normally achieved by allosteric interactions between the protein and DNA, which alters the DNA in a manner that either restricts the interaction of a repressor complex or chromatin structure or permits the interaction of a positive acting factor (22–24). Examples of these allosteric cooperative interactions have been well documented for the structures of the NFAT1·Fos·Jun·ARRE2, MAT alpha 2·MCM1·STE6, and E1·E2 transcription regulatory complexes, which reveal dramatic changes in protein conformation and DNA bending upon complex formation (19–21). Typically, cooperativity is studied between one species of protein that facilitates another protein species binding. However, in the case of AR it is apparent that AR cooperates with other AR molecules to bind to specific arrangements of multiple AREs within a given promoter (9, 14, 15, 18).

The AR binds as a homodimer to individual inverted hexameric DNA half-sites that are spaced by 3 base pairs. Studies have shown that the highest affinity binding for AR and glucocorticoid receptor (GR) occurs on the imperfect palindrome, GGTACAnnnTGTTCT (1, 12). In nature, however, androgen-regulated promoters do not contain this optimized element and instead possess numerous variations of this consensus sequence (reviewed in Ref. 1). In general, these variants are of lower affinity compared with the idealized element, but are

* This work was supported in part by Medical Research Council of Canada (MRC) Operating Grant 14099. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: AR, androgen receptor; ER, estrogen receptor; ARE, androgen response element; GR, glucocorticoid receptor; DBD, DNA binding domain; PR, progesterone receptor; DTT, dithiothreitol; PCR, polymerase chain reaction; DBB, DNA binding buffer; DMS, dimethyl sulfate; RLU, relative luciferase units; MeI, methylation interference; MeP, methylation protection; R1881, synthetic androgen methyl-trienolone.

instrumental in discriminating preferential steroid receptor binding and transcriptional activation (1, 8, 9). Thus there is a wide range of sequences that AR can bind, typically containing the core requirement of three out of four guanines contacts at GGTACAnnnTGTTCT. Sequence variation of the binding site not only affects DNA binding affinity, but also separately affects transcriptional activity in a manner that is discordant with affinity (1).

By extrapolation of crystallographic studies of the GR DNA binding domain (DBD) with DNA, it is assumed that AR uses the same amino acids within its DNA recognition α-helix (CGSCKVFFKRAAE) to form similar hydrogen bonds to nucleotides within the half-sites for sequence-specific recognition (25, 26). The most fundamental contacts obligatorily conserved throughout the nuclear receptor family are between the first lysine, which bonds to the second guanine GGTACA of the half-site and the arginine, which binds to a conserved G base paired to cytosine GGTACA (26–28). These anchoring base contacts are universal within the nuclear receptor family and have been demonstrated for a large number of receptors by a variety of techniques (4, 29, 30). Further discrimination between half-sites is achieved by a specific van der Waals contact between the valine and the thymidine base paired to A, GGTACA for AR, GR, and progesterone receptor (PR) (26–28). The ER binds to an inverted repeat spaced by 3 base pairs with the sequence, AGGTCAnnnTGACCT, by discrimination with its DNA recognition α-helix (CEGCKAFFKRTIQ) using its unique glutamate in a bond to cytosine base paired to the characteristic guanine of an estrogen response element AGGTCA (31). The alanine in the DNA recognition α-helix restricts ER from binding to a sequence containing an AGGACA (7, 32, 33). Although the core consensus half-sites extend for 6 base pairs, other nucleotides are not known to engage in base-specific bonds, but the conservation of these nucleotides imply functional significance. Of interest is the observation that alteration of the sixth nucleotide to a T or G increases transcriptional activity despite lowering DNA binding affinity for AR and PR (1, 34–36). In other studies, it has been shown that particular nucleotides are discriminated against due to incompatibility of a receptor DBD structure and a given sequence, which provides another level of discrimination (1, 7, 25, 37). Therefore, there are general core nucleotide requirements of a steroid response element, but there is also nucleotide variation within the binding site that provides function apart from the receptor binding affinity to the DNA.

AR DNA-binding sites display an exceptional amount of variation in sequence and as isolated binding sites are typically of relatively low affinity and low transcriptional activity. However, it is clear from studies with a number of promoters that physiological levels of response by AR are achieved in conjunction with sets of AREs that are organized into the appropriate spatial architecture (14, 17, 18). How these AREs interact mechanistically in a cooperative manner for DNA binding and in a synergistic manner for transactivation is not understood. In this study we have identified and characterized the nature and mechanism of cooperative binding of AR on androgen-regulated regions of the probasin promoter and the PSA enhancer. We have identified and characterized a subclass of AREs with distinctive sequence variation that AR binds in an allosteric manner altering the local DNA structure. These sites serve as a focal point that facilitates binding to adjacent AREs of more conventional sequence identity. This stabilized complex provides high levels of transcriptional activation by AR and increased AR sensitivity to androgen concentration. This phenomenon appears to be a universal mechanism by which AR interacts cooperatively with DNA to enhance gene expression levels. This furthers our understanding of how androgen-responsive genes are regulated and how the precise nucleotide sequences AREs dictate unique functions.

EXPERIMENTAL PROCEDURES

Recombinant AR DNA Binding Domain Fusion Protein—The rat AR-DBD (amino acids 524–648) was inserted into the *Eco*RI and *Bam*HI sites of pTrcHisC (Invitrogen, San Diego, CA) to construct the six-histidine residue N-terminal fusion protein AR-HisTag. The recombinant protein was expressed in *Escherichia coli* JM109 and purified on nickel-NTA-Sepharose according to the manufacturer's instructions (Qiagen Corp., Germany) except for the following modifications: the column was washed once with 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole, and twice with 50 mM NaH_2PO_4 pH 8.0, 20 mM imidazole. Protein was eluted off the Ni-NTA column with 20 mM Hepes (pH 7.9), 100 mM KCl, 20% glycerol, 1 mM DTT, and 250 mM imidazole. Protein concentrations were determined by Bradford Assays.

Full-length Androgen Receptor—Nuclear extracts were prepared from HeLa cells containing the stable transfection FLAG tag full-length androgen receptor (HeLa-fAR), a kind gift from Michael Carey, UCLA (18). Cells were incubated 24 h in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1 nM R1881 (PerkinElmer Life Sciences) prior to extraction by a method modified from a previous study (18). Briefly, cells were washed twice with phosphate-buffered saline, harvested, and centrifuged 10 min at 1000 rpm. The cell pellet was washed once with ice-cold phosphate-buffered saline and resuspended in 5 volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT), centrifuged 5 min at 1850 $\times$ *g* and resuspended in 3 volumes of buffer A. After 10 min on ice the cells were homogenized with 10 strokes in a Dounce homogenizer, and the nuclei were pelleted by centrifugation for 15 min at 3300 $\times$ *g*. Nuclei were resuspended in 1 volume of buffer C (20 mM HEPES, pH 7.9, 25% v/v glycerol, 20 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT) and incubated at 4 °C for 30 min with gentle mixing. The nuclear extract was obtained by centrifugation at 21,000 $\times$ *g* for 30 min.

Oligonucleotides—Fragments of the rat probasin promoter were generated by PCR amplification using the −286 to + 28 portion of the probasin promoter inserted *Bam*HI/*Hin*dIII into pBluescript (Stratagene, La Jolla, CA) as a template. The fragments studied are as follows: ARE1 G-1 ARE2 G-2 (−269 to −77), G-1 ARE2 G-2 (−229 to −77), ARE1 G-1 (−269 to −164), ARE2 G-2 (−150 to −77), ARE1(−269 to −210), ARE2 (−150 to −105), G-1(−229 to −164), and G-2 (−121 to −77). In addition, six sets of complementary 29-mers corresponding to the wild-type G-1 binding site and five mutant forms were synthesized: wild-type G-1: 5′-CTTATTAGGGACATACCCACAAAT-3′; A195C: 5′-CTTAATAGGGACATAAAGCCCCCAAATAA-3′; C197T: 5′-CTTAATAGGGACATAAAGCTCACAAATAA-3′; G-207A: 5′-CTTAATAGGAACATAAAGCCCACAAATAA-3′; T215G: 5′-CGTAATAGGGACATAAAGCCCACAAATAA-3′; Spacer mutant: 5′-CTTAATAGGGACACGGAGCCCACAAATAA-3′. Oligonucleotides were synthesized by the Nucleic Acid and Protein Services, University of British Columbia, Vancouver, Canada.

DMS Methylation Protection and Methylation Interference—Methylation protection of either the rat probasin promoter and the human PSA enhancer regions was performed by a modified protocol.[2] In brief, histidine-tagged AR-DBD (7.2 μg, 13.6 μM) was incubated at room temperature with 2.0 μg of poly(dI-dC) (Amersham Pharmacia Biotech) and DNA binding buffer (DBB: 20 mM HEPES, pH 7.9, 100 mM KCl, 10% glycerol, 1 mM DTT). To each reaction 350,000 dpm (26.5 fmol) of ^{32}P-single-end-labeled DNA probe was added, and the binding reaction was brought to equilibrium at room temperature. To methylate the protein-bound DNA probe, dimethyl sulfate (DMS) was added to a final concentration of 19 mM and incubated at room temperature for exactly 2 min. The methylation process was stopped by loading the reaction onto a 5% (29:1) polyacrylamide gel containing 0.5× TBE while it was running at 16 V/cm at room temperature. DNA treated in the same manner, but without AR-DBD added, was used as a control.

After separation by polyacrylamide gel electrophoresis, bands indicating protein-bound and protein-free probes were excised and the DNA was eluted. The methylated DNA was cleaved using 1 M piperidine, and the denatured fragments were separated on a 6% (29:1) denaturing polyacrylamide gel containing 1× TBE and 8.3 M urea. Gels were dried and autoradiographed, and the developed images were scanned using a Hewlett-Packard 6300-dpi resolution scanner. Bands were quantitated

[2] Reid, K. J., and Nelson, C. C. (2001) *Biotechniques* **30,** in press

and compared using ImageQuaNT (5.0). Methylation interference of the G-1 and ARE2 fragments of the probasin promoter involved premethylating the DNA fragments before carrying out the binding reaction. The ^{32}P-single-end-labeled DNA probe (200 fmol) in DMS buffer (50 mM sodium cacodylate, pH 8.0, 10 mM $MgCl_2$, and 1.0 μg of calf thymus DNA) was incubated with 45 mM DMS for exactly 2 min at room temperature. The reaction was stopped with DMS Stop Solution (1.5 mM sodium acetate, pH 7.0, and 1.0 M β-mercaptoethanol), and the DNA was ethanol-precipitated. The methylated probe was then used in a protein-binding reaction with AR-DBD as described in the methylation protection protocol. The bands indicating bound and free (in the presence of protein) and unbound (no protein) probes were excised, and the DNA was eluted. The DNA was cleaved using 90 mM NaOH and examined as described in the methylation protection protocol.

Gel Mobility Shift Assays—For gel shift analysis of relative binding affinities, radiolabeled fragments of the probasin promoter were obtained by PCR amplification in the presence of α-dCTP (Amersham Pharmacia Biotech) at 50 mCi/mmol specific activity. Probes were gel-purified as described above. Binding assays were carried out by preincubating AR-DBD-histidine tag protein (10 pmol) with 1 μg of poly(dI-dC) and DBB in a 10-μl volume for 10 min. Increasing amounts of the respective radiolabeled probe were then added in 2 μl of DBB and incubated at room temperature for 10 min. Samples were then loaded onto a 5% polyacrylamide gel and electrophoresed for 90 min at 20 V/cm. The gels were dried and autoradiographed on Biomax MR film (Eastman Kodak Co.). For DNA binding analysis, bands corresponding to the bound protein·DNA complex and free DNA were excised from the dried gel, and the activity of each band was determined by scintillation counting. Relative binding affinity for the probes containing more than one binding site (ARE1 G-1 ARE2 G-2, ARE1 G-1, and G-1 ARE2 G-2) were compared by plotting bound *versus* free values averaged from three independent repetitions for each probe. Binding constants for probes containing one (dimeric) binding site (ARE1, G-1, ARE2, and G-2) were determined by Scatchard analysis as described previously (39).

Transcriptional Activation—Luciferase reporter plasmids were created by introducing *Bam*HI/*Hin*dIII ends to each fragment of the probasin promoter by PCR and cloning into the corresponding restriction sites of pTK-luc (ATCC). LNCaP cells were then transfected with 0.2 μg of reporter plasmid, 1.2 μg of rat androgen receptor in pRcCMV (Invitrogen), and 8 ng of the renilla expression plasmid pRLTK (Promega) using Lipofectin (Life Technologies). Cells were incubated for 22 h in 24-well plates containing either 5% charcoal-stripped medium alone or with R1881 (PerkinElmer Life Sciences), ranging in concentration from 0.001 to 5 nM. Cells were washed and harvested with passive lysis buffer (Promega). Luciferase activity of 20-μl aliquots of lysate was determined using the Dual-Luciferase Reporter assay system (Promega) on a luminometer (Berthold, Germany). Luciferase activity was normalized for transfection efficiency using renilla activity. Experiments were done in triplicate, averaged, and expressed both in relative luciferase units (RLU) and as -fold induction.

RESULTS

DMS Protection Reveals Atypical Androgen Receptor Binding Sites—The AR has been shown to bind to the probasin promoter in a highly cooperative manner (14). Two androgen response elements (AREs) have been previously characterized in the proximal promoter (15). These AREs, referred to as ARE1 and ARE2, are separated by 80 base pairs. If either ARE1 or ARE2 is mutated to a nonbinding form, then binding to the other ARE is impaired and transcriptional activity is greatly compromised (15). In other data, in which the intervening sequence has been deleted, transcription activation by AR is greatly impaired (9). Therefore these data suggest that AR binds cooperatively to the probasin promoter in a functionally significant manner and that this cooperative binding is reliant on the integrity of the intervening sequence (9). To investigate the nature of AR's interaction with the probasin promoter, we employed chemical probe methodologies to investigate the base-specific contacts of AR. To initiate these studies, we used recombinant his-tagged purified AR-DBD extending from amino acids 524 to 648 of the rat AR and DMS-based methylation interference (MeI) to examine the contacts made to guanines by AR. In this methodology the DNA is premethylated prior to binding to the protein of interest. If the methylation of a specific guanine interferes with DNA binding, then the DNA molecule segregates into the unbound or free fraction following gel shift separation and is missing in the corresponding bound fraction. On the other hand, if a methylated guanine does not interfere with binding of the protein, then the DNA molecule appears at equal concentrations in the bound and free fractions. This methodology has been instrumental in indicating DNA-protein contacts to guanines of many bimolecular interactions. However, due to the cooperativity between AR-DBDs bound to the multiple AREs on the probasin promoter, this approach did not produce an interference pattern on the intact promoter, whereas isolated AREs do show MeI patterns (data not shown). This result is most likely, because the prevention of one guanine contact was compensated for by contacts at additional sites and stabilized by protein-protein interactions, a characteristic result we have found of MeI assays throughout these studies when applied to highly cooperative binding sites.

Therefore, we turned to methylation protection (MeP)-based assays in which the DNA·protein complex is formed first and then exposed to DMS to methylate guanines that are not involved in hydrogen bonds with the protein.[2] Protein·DNA complexes are then isolated by electrophoretic mobility shift assay, the bound fraction is isolated and eluted, and methylation patterns are analyzed on a denaturing gel in comparison to control DNA that has not been bound by protein to improve the sensitivity and specificity of DMS protection-based assays.[2] DMS is an ideal chemical modifier probe, because it methylates all guanines equally on free DNA and, therefore, the cleavage patterns produce a uniform ladder of guanines within the sequence. Typically, this approach of MeP provides parallel information to MeI with respect to specific guanine contacts by DNA binding proteins, because they are uniquely protected from DMS attack when the protein is bound and noncontacted guanines are methylated with the same efficiency as in the unbound DNA control. However, if the DNA is locally distorted by binding of a transcription factor, guanines can be hypersensitive to DMS attack (40, 41).

The results from MeP assays of the intact probasin promoter confirmed the previously identified ARE1 and ARE2 and illustrated the expected protection pattern of conserved guanine contacts of the nuclear receptor superfamily with no change in DMS sensitivity to the noncontacted guanine residues within the binding sites ($^{-241}$ATAGCAtctTGTTCT^{-227}) and ($^{-136}$AGTACTccaAGAACC^{-122}) (Fig. 1). However, in addition to these previously recognized binding sites of AR to the probasin promoter, the DMS protection assays revealed two additional potential AR binding sites with atypical half-site sequences located at (−209 to −196) and (−107 to −93). Most intriguingly, these AR binding sites were unusual with respect to their pattern of protected guanines and DMS hypersensitivity. To resolve these complexes of AR-DBD binding to the probasin promoter in more detail, we repeated the MeP assay using smaller fragments of the probasin promoter extending from (−269 to −164) and (−229 to −77) (Fig. 2, *a* and *b*). These higher resolution analyses demonstrated that the guanines in the fifth position of the atypical half-sites were protected as to be expected for nuclear receptor interaction with DNA ($^{-209}$GGGACAtaaAGCCCA^{-196}) and ($^{-107}$ATGACAcaaTGTCAA^{-93}). However, both of the atypical sites had guanines in the third position of the presumed 5′-half-sites that showed dramatic DMS hypersensitivity and even more pronounced DMS hypersensitivity in the 3′-half-sites ($^{-209}$GGGACAtaaAGCCCA^{-196}) and ($^{-107}$ATGACAcaaTGTCAA^{-93}). Hypersensitivity to DMS methylation is most commonly attributed to structural alteration of the DNA, including strand displace-

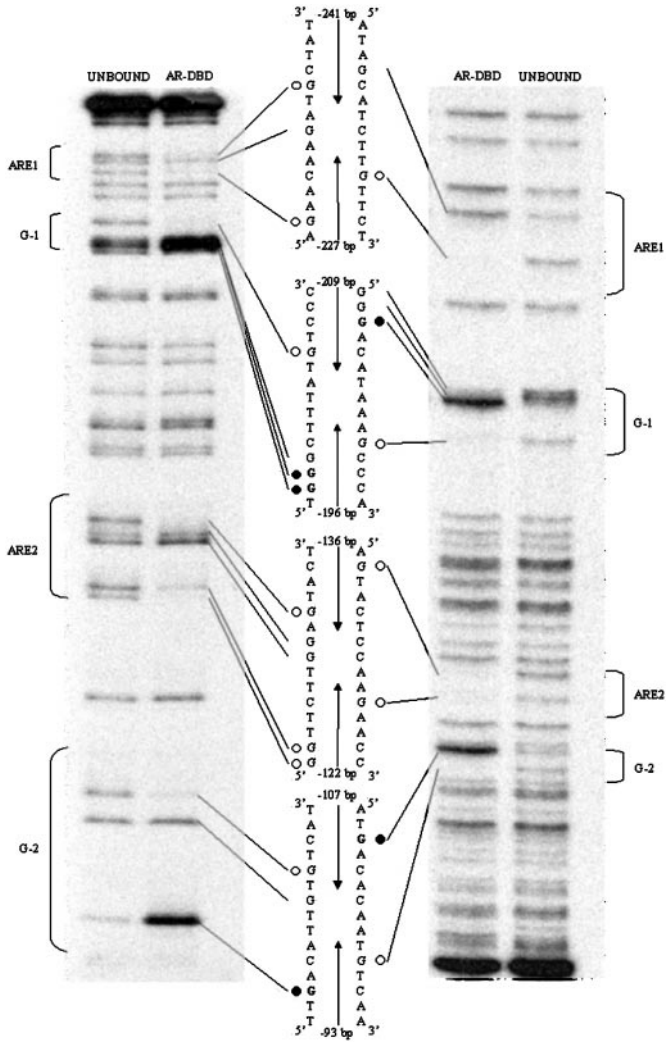

FIG. 1. **The probasin reveals four AR-DBD-binding sites using methylation protection assay.** The AR-DBD was bound to the probasin promoter (−269 to −77) and analyzed by DMS-based methylation protection. Class I binding sites, ARE1 and ARE2, and class II binding sites, G-1 and G-2, are illustrated with their sequences. *Arrows* indicate half-site location and orientation. *Open circles* represent protected guanines residues, whereas *solid circles* represent guanines hypersensitive to DMS.

ment, kinking, and other forms of local DNA structure deformation (42, 43). According to our understanding of the binding of nuclear receptors to their response elements, the DNA is in B-form and the guanine in the second location of the half-site AGCCCA should be protected, not overtly hypersensitive (26, 28, 31, 44). The most striking consistent feature of these newly identified AR-binding sites is that the hypersensitive guanines are located in the position of the half-site that is a characteristic discriminatory nucleotide within estrogen receptor (ER)-responsive binding sites ($^{-209}$GGGACAtaaAGCCCA^{-196}) and ($^{-107}$ATGACAcaaTGTCAA^{-93}) (27, 31, 45, 46). However, the conservation of the adenine adjacent to the guanine on these binding sites prohibits interaction with ER (7, 32) (data not shown). Interestingly, in a previous study of binding site selection, we have shown that a guanine at this location is preferred for PR and equally preferable to the consensus thymidine for AR (1).

Because of the unusual nature of these AR-binding sites containing hypersensitive guanines in the third position of the half-site, we have referred to these individual sites within the probasin promoter as G-1 and G-2. The unusual binding patterns of G-1 and G-2 with respect to DMS hypersensitivity have lead us to classify these AR interactions as class II type binding in contrast to the typical binding pattern hereafter called class I type binding as demonstrated on ARE1 and ARE2.

Binding of Class II Sites Is Independent of Adjacent Class I Sites—The apparent binding of the AR-DBD to the class II G-1 and G-2 sequences could be a function of weak interactions that are buttressed by the two class I AR-DBD homodimers bound to ARE1 and ARE2, respectively. If these AR dimers physically interact in a cooperative manner, the intervening DNA must assume a loop structure. Therefore, it is possible that the interactions of class I AR dimers on ARE1 and ARE2 could alter the DNA conformation such that further interactions of the class I dimers to distant sites are permitted. This could result in DNA torsional strain causing the guanine hypersensitivity seen on the G-1 and G-2 regions. Alternatively, G-1 and G-2 could be bound independently by two additional AR homodimers and the hypersensitivity of these sequences may be intrinsic to the nature of AR-DBD binding to this complex promoter with four interacting sites. To distinguish between these two possibilities the four individual binding elements were tested in isolation for their ability to bind to AR. These four individual fragments all bound AR-DBD and were each further characterized for DNA binding affinity by Scatchard analyses. These analyses were performed using a constant amount of purified AR-DBD and increasing concentrations of radiolabeled DNA in an electrophoretic mobility shift-based assay to avoid inconsistencies caused by protein dilution over large ranges of concentration. These data demonstrated that the relative DNA binding affinities of the AR-DBD were 6.3 nM for ARE2, 11 nM for ARE1, 14 nM for G-1, and 18 nM for G-2. Although the isolated class I sites have slightly higher affinity than the class II sites of the probasin promoter, all elements displayed appreciable binding activity.

Because it was demonstrated that G-1 and G-2 were independent binding sites of AR-DBD homodimers, we next wanted to determine whether the DMS hypersensitivity seen on the G-1 and G-2 elements was an intrinsic property of the individual G-1 and G-2 primary DNA sequence or if hypersensitivity was a result of the multiple complex interactions in the context of the promoter containing AREs interacting in a highly cooperative manner. Using the MeP assay as described above, it was found that the relative pattern of protection and hypersensitivity of AR binding to the isolated G-1 and G-2 elements was highly similar to the pattern observed when this technique was applied to the entire probasin promoter or its fragments (Fig. 3). Full-length flag-tagged AR prepared from a stably transfected HeLa cell line also demonstrated the hypersensitive pattern seen with the AR-DBD (data not shown) (18). To investigate further how the hypersensitive guanines were involved in binding to the class II sites, we performed MeI in which the DNA is premethylated as discussed above. In the MeI analysis we found that the premethylation of the guanines prevented binding by AR to both class I and class II binding sites (Fig. 3). We interpret this intriguing set of findings to mean that the guanines initially involved in hydrogen bonds with AR in the recognition of the class II binding sites, as they are in class I sites. However, after this initial recognition interaction, there is an allosteric conformational change to the class II binding sites in which the interaction with guanines is altered and becomes hypersensitive to methylation. In summary, these results demonstrate that G-1 and G-2 can bind AR independently, likely in an allosteric manner and that the specific nucleotide sequence of these class II elements dictates the hypersensitivity to guanine methylation within their half-sites.

Class II Type Binding Sites Nucleate the Cooperative Binding

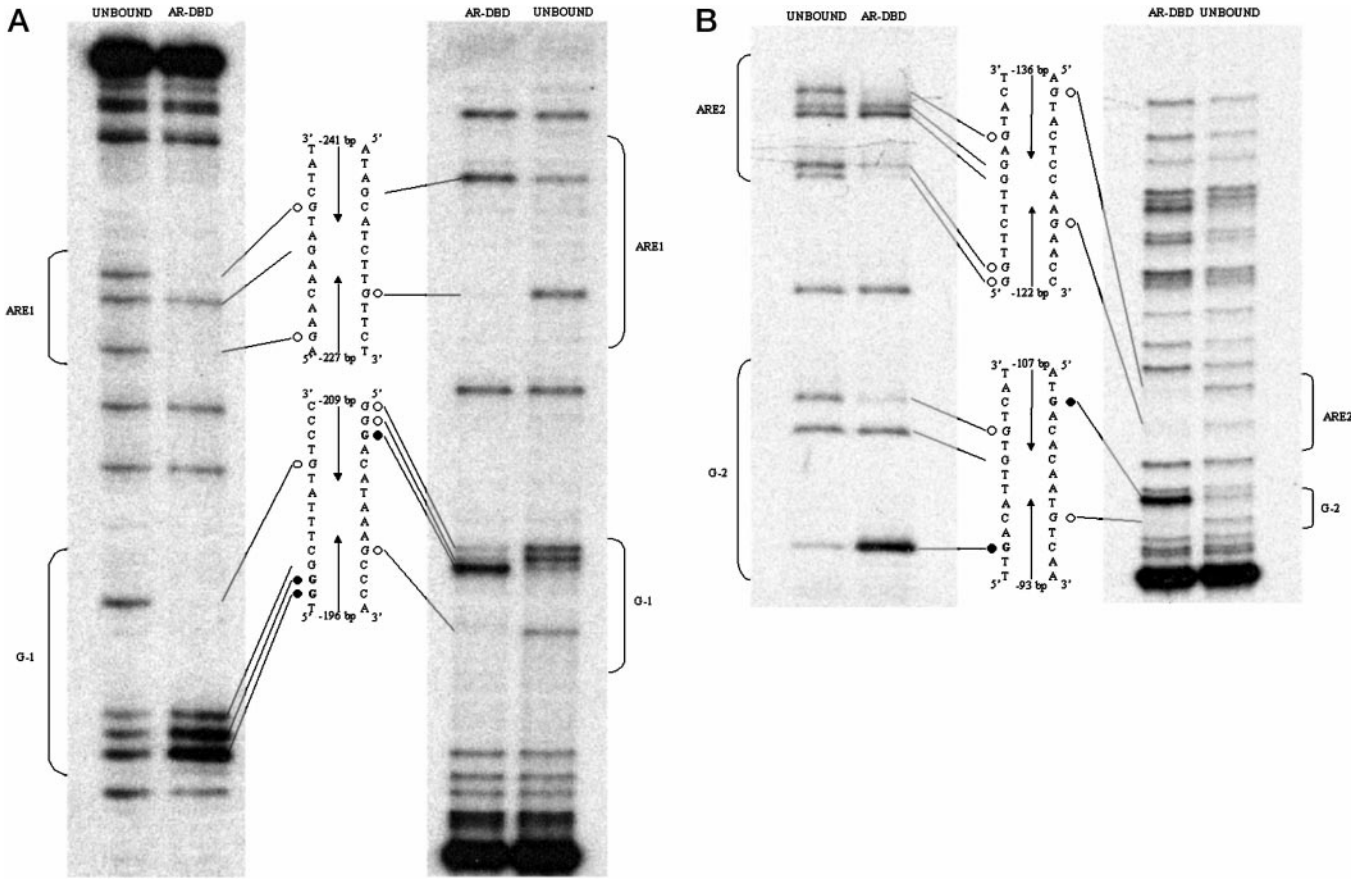

FIG. 2. **Pronounced DMS hypersensitivity in the 5′- and 3′-half-sites of class II AR-binding sites on the probasin promoter.** The probasin promoter fragments from (−269 to −164) and (−181 to −77) were bound by AR-DBD and assayed by methylation protection. *Arrows* indicate half-site orientation and location. *Open circles* represent protected guanines, and *solid circles* represent hypersensitive guanines. *A*, class I binding element, ARE1, and class II binding element, G-1, are illustrated with their nucleotide sequence. *B*, class I binding element, ARE2, and class II binding element, G-2, are illustrated with their nucleotide sequence.

to Adjacent Class I Sites—To investigate the functional role of these class II binding sites in terms of cooperative binding by AR to the probasin promoter, we created a series of DNA fragments of various regions of the promoter containing combinations of ARE1, G-1, ARE2, and G-2 in their native context. We analyzed combinations of the class I and class II elements to determine the relative contribution of individual binding sites to cooperativity as judged by complex formation as a function of increasing DNA concentration as was performed above for singular elements (Fig. 4) (39). These analyses revealed that the combination of ARE1 and G-1 (with K_d values of 11 and 14 nM in isolation, respectively) resulted in a cooperative interaction that half-saturated the DNA at more than a 10-fold lower concentration (0.9 nM). Similarly ARE2 and G-2 interacted in a cooperative manner to shift the rate of complex formation approximately an order of magnitude lower in concentration. The promoter fragment containing G-1, ARE2, and G-2 interacted with the highest degree of DNA binding cooperativity resulting in half-saturated binding at 0.11 nM, which is over 50 times stronger binding than ARE2, the individual element with the highest DNA binding affinity. Interestingly, the addition of ARE1 to this extremely cooperative DNA fragment of G-1, ARE2, and G-2 resulted in weakening the overall strength of the DNA binding complex with all four sites occupied to ~1.2 nM to achieve half saturation. Comparable binding results were observed with increasing AR-DBD protein concentration in the presence of a constant amount of DNA. In thorough examination of AR binding to this promoter containing all four binding sites, it was apparent that the binding curve was biphasic. The order of complex formation appears to be occupation of G1-ARE2-G2 at low concentrations of protein, followed by additional binding to ARE1 as protein concentration increases. These results indicate that class II elements are instrumental in providing the dramatic level of DNA binding cooperativity seen on the probasin promoter. It is also clear that the four individual elements interact in a complex manner in which G-1 can interact individually with ARE1 or ARE2-G2. However, if both ARE1 and ARE2-G2 are present on the same DNA fragment, then the interaction between G-1 and ARE2-G2 is weakened, presumably, through an interaction with ARE1, thereby affecting the overall stability of the complex.

Contribution of Class I and Class II Sites to Transcriptional Activation on the Probasin Promoter—To determine whether class II AR-binding sites contribute to the transcriptional activity of the probasin promoter in response to hormone induction, we tested combinations of ARE1, G-1, ARE2, and G-2 as well as the individual class I and class II elements for transactivation potential in the presence of increasing concentrations of the synthetic androgen R1881 in LNCaP cells. All individual class I and class II sites displayed low levels of transcriptional activity (less than 2000 RLUs) that were maximal at 0.5 nM R1881corresponding to levels of 12-, 23-, 25-, and 33-fold, for G-1, ARE1, G-2, and ARE2, respectively (Fig. 5*a*). Combining class I and II sites resulted in an increase in the sensitivity to hormone concentration maximal at ~0.05 nM R1881, but the magnitude of transcriptional activation of ARE1-G1 and ARE2-G2 was at most additive of the activity of the individual elements (Fig. 5*b*). The addition of G-1 to the ARE2-G2 binding element resulted in dramatic synergistic transcriptional activation to a maximum of 169-fold induction

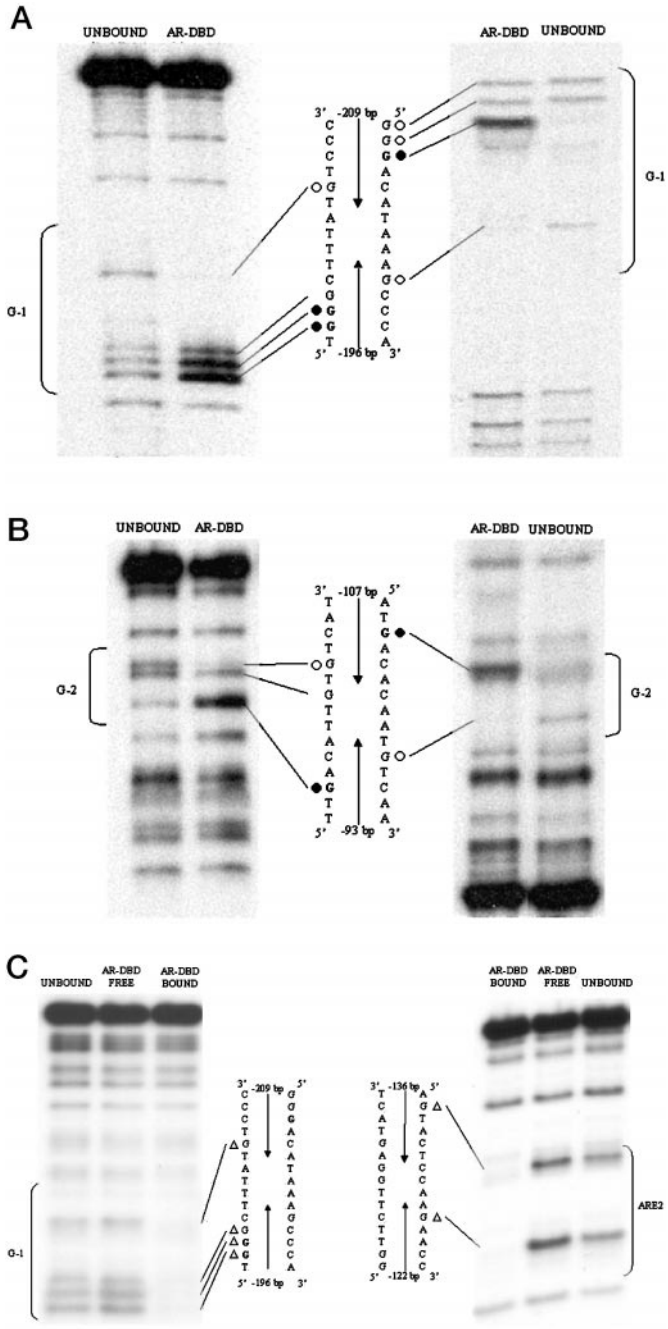

FIG. 3. Class **II binding elements demonstrate intrinsic DMS hypersensitivity upon AR-DBD binding.** Methylation protection assays on individual class II AR-binding sites. *Arrows* denote half-site orientation and location for each binding element sequence. *Open circles* represent protected guanines, whereas *solid circles* represent hypersensitive guanines. *A*, methylation protection of class II AR-binding element G-1 contained within −231 to −164. *B*, methylation protection of class II AR-binding element G-2 contained within −128 to −56. *C*, methylation interference assay demonstrates that conventional guanine contacts are necessary for AR binding to G-1 and ARE2.

(~20,000 RLUs), consistent with the increase in DNA binding cooperativity when G-1 is combined with ARE2-G2 (Fig. 5*c*). The addition of ARE1 to the highly cooperative DNA binding region containing G-1, ARE2, and G-2 resulted in a biphasic curve from 0.01 to 0.05 nM followed by a sharp increase in level of activation to culminate in a 5-fold enhanced transcriptional response (~100,000 RLUs) compared with G-1-ARE2-G2 alone and more than 30-fold greater than any individual element (Fig. 5*c*).

The higher relative transcriptional activity of ARE1-G1-ARE2-G2 is in contrast to the cooperative binding data, which demonstrated that the addition of ARE1 decreased the overall binding strength of the complex. Together these data suggest that a biphasic curve of activity arises from the highly cooperative DNA binding complex at low concentrations of R1881, presumably corresponding to concentration of activated AR in the nucleus, whereas, at high levels of R1881 (activated AR), the inclusion of ARE1 is able to provide highly synergistic levels of transcriptional activation. In summary, these data suggest that unique combinations of class I and class II sites are required for maximal transcriptional activation by AR and that each class I and class II element plays a unique functional role in transcriptional activation, DNA affinity, and complex stability that in a composite fashion culminates in a high level of transcriptional induction. Furthermore, these results illustrate that androgen-regulated promoters can respond in a rheostat fashion to hormone concentration generally reflecting the degree of cooperative DNA binding to the given promoter.

Characterization of class II Binding Sites of AR on the PSA Enhancer—The AR has been reported to bind cooperatively to a number of androgen-regulated promoters (9, 17, 18, 38). To determine whether the principles of class I and class II AR binding sites that we have documented for the probasin promoter also applied to other cooperative promoters, we investigated the characteristics of AR binding to the PSA enhancer. This enhancer has recently been characterized in detail, identifying four AR binding sites, referred to in the earlier study as V, IV, III, and IIIa, which interact in a cooperative manner, and each site contributes to full androgen induction (18). To determine whether this cooperatively bound enhancer of the *PSA* gene contained class II binding sites for the AR, we used DMS protection assays as described for the probasin promoter. This analysis demonstrated that two of the previously characterized AREs, V, and IIIa, within the PSA enhancer had the same distinctive features of hypersensitivity reported above for the probasin promoter (Fig. 6). The high affinity PSA enhancer site III was similar to a class I element. The PSA element, IV, was similar to the class II sites but does not possess enough guanines to confidently classify this element. From this analysis of the PSA enhancer it was evident that additional guanines immediately flanking the half-sites present only in the PSA enhancer sequence showed hypersensitivity to DMS. Therefore, this analysis provides additional information that the structural distortion induced by AR binding to these class II sites may extend over an area of at least 17 base pairs.

Consensus Sequence for Hypersensitive class II Sites of AR—To determine the consensus features of class II binding sites of AR, the elements that displayed the characteristic hypersensitive guanines within the probasin promoter and PSA enhancer were aligned to identify similarities. The numbering scheme for this analysis refers to the reference strand with the base location indicated in reference to the central nucleotide of the palindrome as 0 and ascending toward the 3′-half-site and descending toward the 5′-half-site (Fig. 7). This comparison of AR-binding sites within the PSA enhancer and probasin promoter derived a unique consensus of distinctive features that distinguish class I and class II sites. Both class I and II sites have a Cys at −3 and a Gly at +3. Class II sites uniquely have a Gly at −5 and a Cys at +4 and/or +5. The most prominent feature of class II sites is a purine at +7, which displays a highly conserved pyrimidine in class I sites. Interestingly, class II sites also contain a Tht at position −13 in all cases and a corresponding Ala at +13 in all but one case. Additionally, there is a tendency to have a higher AT-rich flanking region and spacer in class II sites in comparison to class I sites.

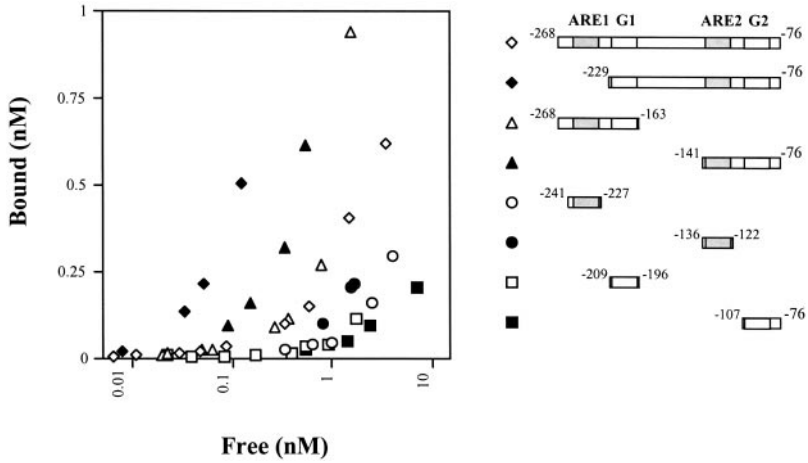

FIG. 4. Class **II AR-binding sites facilitate DNA cooperative binding on the probasin promoter.** Relative binding affinities of probasin promoter elements were determined by gel shift assay using 5 nM purified His-tagged AR-DBD with increasing concentrations of radiolabeled DNA as indicated. Bound and free fractions were isolated using a gel shift assay, excised, and counted. The change of the bound over free ratio is plotted as a function of increasing input DNA. Only values corresponding to the linear portion of the binding curve are given. ARE1, *open circles*; ARE2, *filled circles*; G1, *open squares*; G2, *filled squares*; A1-G1, *open triangles*; A2-G2, *filled triangles*; G1-A2-G2, *open diamonds*; A1-G1-A2-G2, *filled diamonds*.

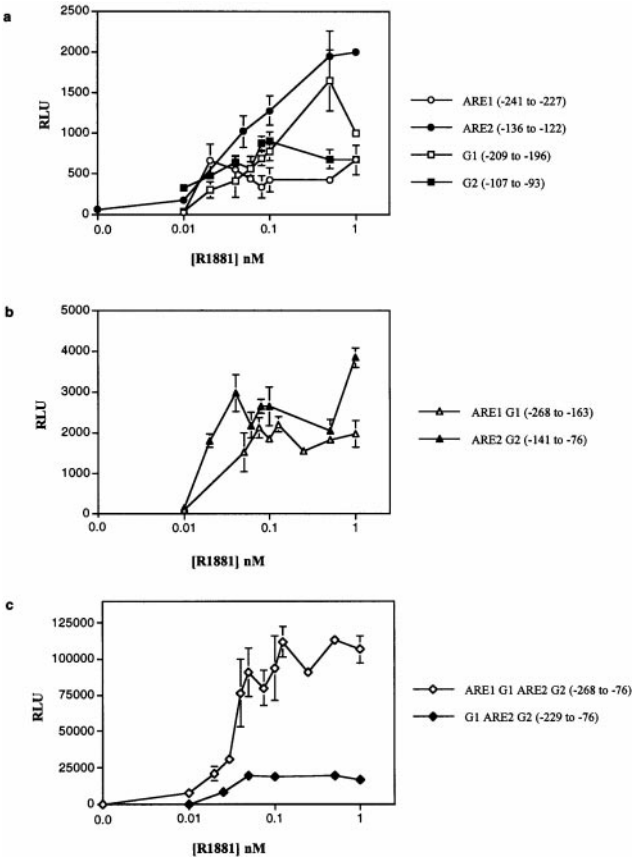

FIG. 5. **Class I and class II AR-binding sites display low levels of transcriptional activity in isolation and interact synergistically in combination.** *a*, transactivation activity of individual elements was tested in the presence of increasing concentrations of R1881. ARE1 (*open circles*), ARE2 (*filled circles*), G-1 (*open squares*), and G-2 (*filled squares*). The boundaries of each site within the promoter are indicated in Ref. 43. LNCaP cells transfected with pTK-luc reporter plasmids containing the fragments were incubated 22 h with the indicated concentration of R1881 then assayed for luciferase activity. Transcriptional activity is reported as relative luciferase units (RLU) corrected for background. *b*, effects on transactivation activity of combining class I and class II AR binding sites of the probasin promoter as assayed in *A*. Levels for ARE1-G1 (*open triangles*), ARE2-G2 (*filled triangles*) are shown. *c*, transcriptional activity of multiple class I and class II AR binding sites of the probasin promoter, G-1 ARE2 G-2 (*closed diamonds*) and ARE1 G-1 ARE2 G-2 (*open diamonds*) were tested as described above.

Sequence Determinants for DMS-hypersensitive AR-binding Sites—Because class II sites appeared to have distinctive consensus nucleotides and demonstrated atypical AR binding patterns in isolation, we next investigated the nucleotide determinants of the class II sites that result in the allosteric structural changes upon AR binding. To do so, we focused on the G-1 binding site from the probasin promoter using site-directed mutagenesis followed by analysis of the DMS protection patterns after binding of AR. The distinctive features of the class II type binding sites in comparison to class I sites, other than the aforementioned guanines in the third position of the half-site, is that the spacer region was relatively AT-rich in comparison to class I type AREs. In addition it was noted that class II sites had a Thr 6 base pairs 5′ of each half-site and that in the 3′-half-site the sixth nucleotide was a purine in contrast to the consensus pyrimidine in this location of class I sites. These individual mutations were tested for their potential contribution to hypersensitivity as before (Fig. 8). The results from DMS protection assays demonstrated that conversion of the Thr at position −13, six bases 5′ of the half-site, decreased the guanine hypersensitivity in the 5′-half-site but did not affect the binding pattern of the 3′-half-site. Likewise, conversion of the half-site spacer nucleotides from TAA to CGG also decreased the hypersensitivity in the 5′-half-site alone. When the Ala at position +7 of the 3′-half-site was converted to a Cys, the DMS protection binding pattern of the 3′-half-site reverted to the pattern observed with a class I type binding site but did not alter the hypersensitivity in the 5′-half-site. Therefore, these results suggest that class II type binding by AR is a composite of influences, primarily distinguished by the nonconsensus purine nucleotide at the +7 position of the 3′-half-site, but is also influenced by the identity of other nucleotides in the flanking and spacer regions.

In summary, these analyses clearly demonstrate that AR binds to two structurally and functionally distinct classes of AREs that are directed by allosteric interactions of the binding complex. AR binding to class I sites have been previously recognized and use conventional nucleotide contacts used by most nuclear receptors. These are of low DNA binding affinity and transcriptional activity in isolation. In contrast, class II binding sites result in DNA structural alterations that display DMS hypersensitivity to particular guanines expected to be contacted, but are also of low affinity and transcriptional activation as individual elements. Unique combinations of class I and class II sites result in dramatic cooperative DNA binding affinity and a highly synergistic effect upon transcriptional activity. This complex, composite function that facilitates cooperative DNA binding and achieves a higher level of transcriptional response is dictated by the primary nucleotide sequence to which the AR binds.

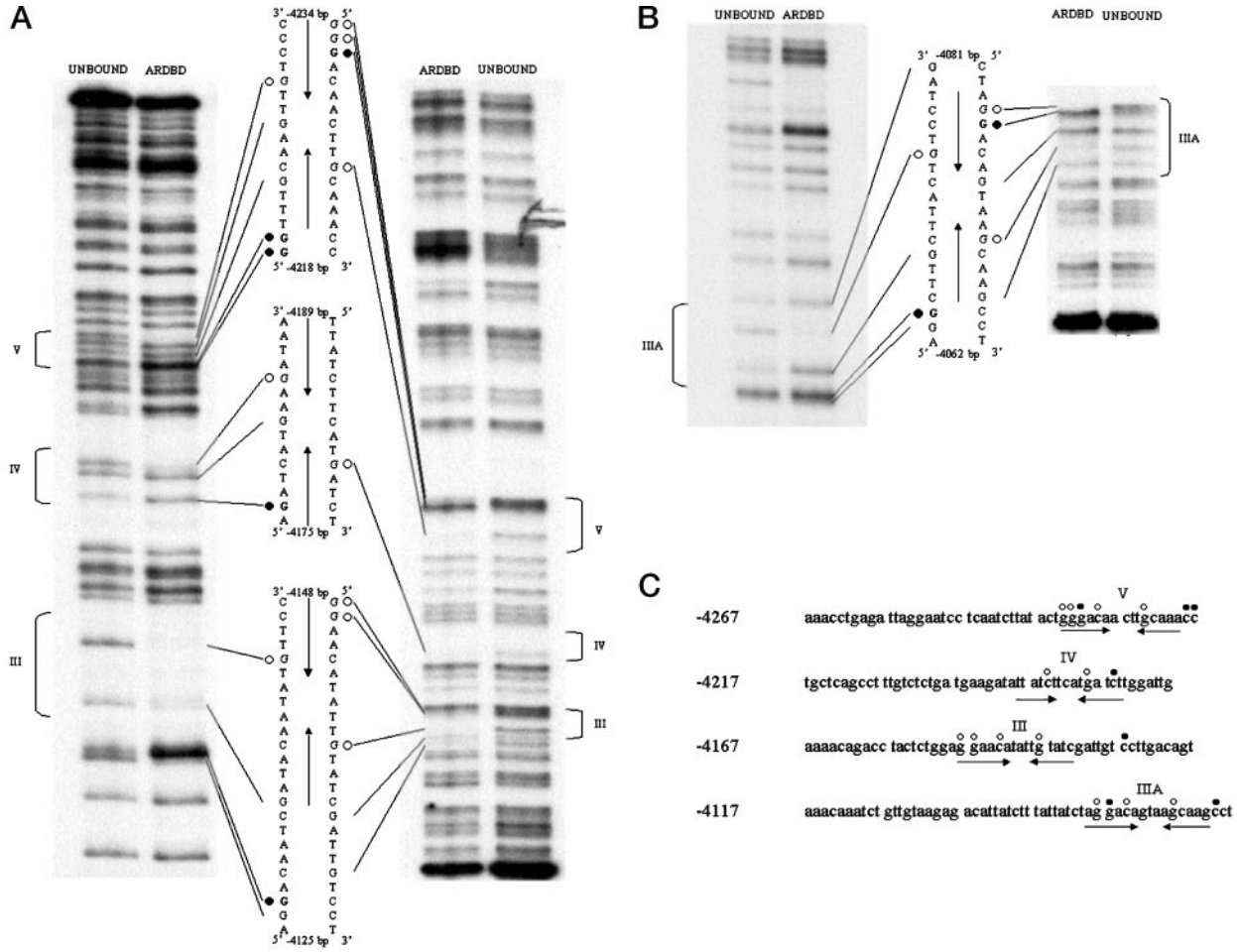

FIG. 6. **The PSA enhancer (−4366 to −3874 bp) has three class II AR-binding sites as revealed by methylation protection analysis.** In all panels, *open circles* represent protected guanines, whereas *solid circles* represent hypersensitive guanines. *Arrows* denote half-site orientation within each ARE. *a*, the AR-binding site III has a class I protection pattern, whereas AR-binding site V has similarity to class II type protection/hypersensitive patterns. *b*, AR-binding site IIIA has a class II protection/hypersensitive pattern. *c*, the locations of the class I and class II sites within the PSA enhancer sequence are indicated.

Probasin G-1	-209 GGGACA-TAA-AGCCCA -196
Probasin G-2	-107 ATGACA-CAA-TGTCAA -93
PSA Enhancer V	-4234 GGGACA-ACT-TGCAAA -4220
PSA Enhancer IIIA	-4079 AGGACA-GTA-AGCAAG -4065
PSA Enhancer IV	-4175 AGATCA-TGA-AGATAA -4189
SLP 2	+142 *AGAACT-GGC-TGACCA* +128
CONSENSUS CLASS II	RGGACA-NNA-AGCCAA
	-7 -2 0 +2 +7
CONSENSUS CLASS I	RGAACA-NGN-TGTNCT
Probasin ARE1	-241 ATAGCA-TCT-TGTTCT -227
Probasin ARE2	-136 AGTACT-CCA-AGAACC -122
PSA Enhancer III	-4148 GGAACA-TAT-TGTATT -4134
PSA ARR	-390 *GGATCA-GGG-AGTCTC* -376
PSA ARE	-167 *AGAACA-GCA-AGTGCT* -153
SLP 3	+144 *AGAACA-GGC-TGTTTC* +158

FIG. 7. **Alignment of AREs into proposed class II and class I AR-binding sites.** ARE sequences from the probasin promoter, PSA enhancer, PSA promoter, and *Slp* gene. Consensus features are highlighted by *solid circles* for class II and open circles for class I, with conserved guanine contacts *underlined*. *Italicized* elements are proposed by sequence similarity to the class designation.

DISCUSSION

The AR can bind to a large repertoire of sequence variants that resemble an inverted palindromic repeat spaced by 3 base pairs. The sequence identity of the individual AR binding sites within natural promoters may be incidental or may impart function to the receptor response. In a previous analysis of AR binding sites, we demonstrated that some of the nucleotide variation within AR binding sites aid in discrimination of binding by GR and PR (1). Likewise, some nucleotide substitutions within the binding site affected transcriptional activity in a manner that was discordant with their effects on DNA binding affinity (1, 34–36). Thus, the nucleotide sequence of an ARE can separately function to help discriminate both preferential steroid receptor binding and transcriptional activation. Our previous study also provided evidence that AR binds to a nearly palindromic DNA binding site in an asymmetrical manner that extends 5 base pairs away from the previously recognized target. Together this suggests that AR binds to the DNA in an allosteric manner and that the nucleotide sequence of the response element imparts functional information to the AR-protein to which it is bound.

In the present study we have investigated the nature in which AR binds cooperatively to multiple AREs within androgen-regulated promoters. This study has led to the identification of two distinct classes of AR binding sites. Class I sites display conventional binding patterns in terms of guanine contacts. Class II are distinguished by their unusual patterns of hypersensitivity seen by DMS protection assays in response to interaction with AR. Class II sites function to facilitate cooperative binding to adjacent class I sites, presumably through local DNA structural alterations. Both class I and class II sites in isolation are of low binding affinity and low transcriptional

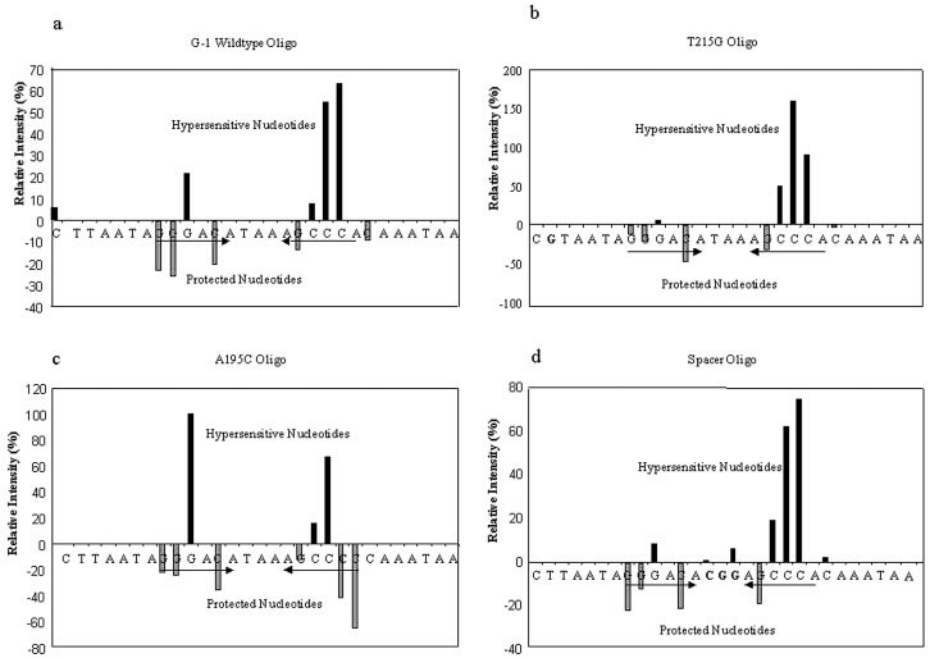

FIG. 8. **Nucleotide determinants of class II AR-binding sites as determined by site-directed mutagenesis.** To quantitate the relative protection or hypersensitivity of each guanine band the band intensity of bound AR-DBD to the probasin G-1 wild-type, A195C, T215G, or SPACER oligonucleotides indicated was compared with the unbound oligonucleotide using ImageQuaNT 5.0 densitometry analysis. Hypersensitive guanines are shown with positive intensity relative to the unbound band and are illustrated using *black bars*. Protected guanines are shown with negative intensity relative to the unbound band and are illustrated using *gray bars*. ARE half-site orientation and location are indicated by *arrows*. *a*, the wild-type G-1 oligo shows the typical class II methylation pattern. *b*, changing the upstream conserved thymidine at position-13 to a guanine decreased the hypersensitivity on the 5′-half-site at position −5. *c*, mutating the 3′-half-site such that it more resembles a class I half-site by changing the adenine at position +7 to a cytosine resulted in an overall decrease in hypersensitivity in the 3′-half-site and a newly protected cytosine at position +6. *d*, changing the ATT spacer region to a CGG decreased the hypersensitivity on the 5′-half-site at position −5.

activity, requiring over 0.5 nM R1881 for full induction in transient transfection assays. Whereas, specific combinations of class I and class II sites found in androgen-regulated promoters synergistically increase DNA binding affinity, hormone sensitivity, and levels of transcription in comparison to singular AREs. The degree of DNA binding cooperativity most strongly correlates to the high levels of transcriptional induction at very low levels of hormone.

Summarizing the data obtained in this study and other studies of the probasin promoter, it is most likely that G-1, ARE2, and G-2 interact cooperatively to increase the stability of the DNA-binding complex and correspondingly are activated at relatively low levels of hormone concentration. At higher levels of androgens, this most highly cooperative complex for DNA binding may be poised to stabilize binding of an additional AR dimer to ARE1. The additional interaction with ARE1 decreases the physical stability of the overall complex, but provides maximal transcriptional activation.

Our working hypothesis is that the AR dimer bound to ARE1 may interact most efficiently with coregulator proteins to induce androgen-dependent transcription from this promoter, whereas ARE2 in this context is more integrally involved in protein-protein interactions with AR molecules bound to G-1 and G-2. In the highly stable G-1·ARE2·G-2 complex, the interaction site for AR coregulators of the three homodimers may be less accessible. Therefore, when ARE1 is mutated, transcriptional activity is lost possibly due to a lack of efficient coactivator interactions with the remaining complex, whereas when ARE2 is mutated, transcriptional activity is greatly decreased due to impairment of DNA binding cooperativity (14). Thus each of the four elements imparts a different function that cumulates in a high level of transcriptional activity. In contrast, when these elements are bound by AR in isolation of other AR sites, they exhibit a low level of transcriptional activity, and only at high levels of hormone, compared with the highly cooperative DNA-binding complexes, which are activated at much lower concentrations of hormone. We interpret this shift in the concentration of hormone induction to be a function of titration of activated AR to the nucleus. Highly cooperative AR complexes would require a correspondingly lesser amount of activated AR for activation of transcription, as paralleled by the cooperative DNA binding data. This study demonstrates that the DNA-binding sites respond in a sequence- and concentration-dependent manner to the binding of AR and that AR reacts differentially, in terms of function, to binding DNA dependent upon the nucleotide sequence. This evidence strongly suggests that the AR interaction with DNA is not an inert binding process or docking station but is in fact mutually allosteric in a functionally significant manner. This model gives credence to the hypothesis that sequence variation of AREs is not incidental in natural promoters, but imparts function to the response to hormone induction in a complex composite manner.

In examination of other androgen-regulated promoters that interact with the AR in a cooperative manner, we have identified putative class II binding sites, which suggests that this is a universal mechanism by which the AR binds to multiple AREs in a cooperative manner. In building on previous work and the studies reported here we hypothesize that DNA sequence dictates function to the AR that is mediated by sequence-specific allosteric interactions. These mechanisms, when used in combination, may provide for a rheostat of androgenic response both in terms of amplitude and duration, thereby allowing a specific gene to customize the transcriptional response to a hormone stimulus in a promoter/gene-specific fashion.

Acknowledgments—We are appreciative of many helpful discussions with Drs. Paul Rennie, Robert Matusik, and Susan Kasper. C. Nelson

gratefully acknowledges her support as a Medical Research Council of Canada Scholar.

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Two Classes of Androgen Receptor Elements Mediate Cooperativity through Allosteric Interactions

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J. Biol. Chem. 2001, 276:2943-2952.
doi: 10.1074/jbc.M009170200 originally published online October 30, 2000
